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I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2002951270 for a patent by VRI BIOMEDICAL LTD as filed on 06 September 2002.

WITNESS my hand this
Nineteenth day of September 2003

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AUSTRALIA

PATENTS ACT 1990

PROVISIONAL SPECIFICATION

FOR THE INVENTION ENTITLED:-

" PROBIOTIC BACTERIUM AND METHODS OF USE"

The invention is described in the following statement:-

TECHNICAL FIELD

The present invention relates to variants of the bacterium *Lactobacillus fermentum*, formulations of the variant and its uses in preventing and/or treating diseases of mammals, and promoting mammalian health.

BACKGROUND

Indigenous bacteria play a major role in preventing certain bacterial and fungal diseases. They do this by a process of bacterial antagonism, preventing other microbes from establishing a presence in the body. Mechanisms involved in this activity include direct competition for nutrients, alterations in the acidity of an area making it hostile for other microbes, producing inhibitory metabolites and anti-microbial chemicals and by preventing other microbes from attaching to host surfaces.

Indigenous flora also influences the immune system. Animals reared in germ free environments exhibit underdeveloped and relatively undifferentiated lymphoid tissues, low levels of immune proteins and a primary response to infection rather than a pronounced secondary response.

These microbes play an important role in the health of the host, by producing essential vitamins and nutrients required by the colonocytes, assisting with the degradation of complex nutrients, protecting the host from invasion by pathogens and stimulating the immune system. In addition, it has been proposed that the microbes in the bowel contribute to mineral absorption and lipid metabolism because of the lowered pH as a result of the short chain fatty acids produced by the microbes. These short chain fatty acids are regulators of colon physiology and play an important role in maintaining normal bowel function.

The upper gastrointestinal tract contains only bacteria swallowed with the saliva and food. As a result of the high acidity of gastric juices few organisms, mostly lactobacilli, can be cultured from the normal stomach. The upper small intestine contains relatively sparse numbers of lactobacilli

and enterococci. The flora of the gastrointestinal tract gradually changes until it becomes similar to that found in the colon, predominantly *Bacteroides, Bifidobacterium, Fusobacterium, Lactobacillus* and *Eubacterium.* These microbes are known as strict anaerobes, in that the presence of oxygen will kill them and they obtain their energy by fermentative processes.

This normal flora of the body is a complex ecosystem, which is regulated by the diet, microbial interactions and host factors such as the motility of the gut and intestinal secretions. External factors such as stress, dietary changes and medications can affect the normal flora and alter the types of organisms present or their metabolism. If the balance is disrupted it can be detrimental to the host and can cause disease.

Sometimes however the balance is lost and invading pathogens are successful in penetrating the body's defences causing infections or triggering inappropriate immune responses. Similarly the balance between host and indigenous flora can be compromised leading to infection by normally dormant organisms, e.g. episodes of thrush (*Candida albicans* infection) following the use of antibiotics.

Disappointingly the "magic bullets" that antibiotics apparently offered have lost some of their impact. In the last 30 years an ever-increasing problem of antibiotic resistance has become a major public health issue. Today some antibiotics are all but useless against certain microbes, while some bacteria are resistant to almost all known antibiotic medications.

Further, evidence is accumulating that the indigenous microbes of the large bowel may be implicated in irritable bowel syndrome (IBS), a condition characterized by changes in bowel habit, disordered defaecation and distension.

Clearly alternative and perhaps complementary method of treatment are required.

It is an object of the present invention to overcome or ameliorate at least one of the disadvantages of the prior art, or to provide a useful alternative.

SUMMARY OF THE INVENTION

The new probiotic bacterial variant, *Lactobacillus fermentum* variant VRI 003 has surprising advantageous features over pre-existing strains of this bacterium as well as other probiotic microorganisms. It is particularly useful in the prevention and/or treatment of gastrointestinal disorders due to its advantageous ability to colonise the gastrointestinal tract. Further, this variant has advantageous immunomodulatory effects, which occur both locally in the gut, and at mucosal sites throughout the body via the common mucosal immune system.

According to a first aspect of the invention, there is provided a novel *Lactobacillus fermentum* variant strain VRI 003 (accession number NM02/31074).

According to a second aspect of the invention, there is provided a composition comprising a *Lactobacillus fermentum* variant according to the first aspect, or components thereof, and a pharmaceutically acceptable carrier.

Preferably, the composition may be prepared as tablets, capsules, powders, or liquid formulation and the like. The *Lactobacillus fermentum* variant VRI 003, or the composition containing said variant, may be administered by any known means but preferably it is administered orally. The variant VRI 003, or the composition containing said variant, may also be advantageously administered topically, either by application to a mucosal surface, or by application directly to the affected skin areas.

According to a third aspect, the invention provides a dietary supplement or a food preparation comprising *Lactobacillus fermentum* variant VRI 003, or components thereof.

Preferably the food product is a dairy or dairy-based food product.

Lactobacillus fermentum variant VRI 003, or the composition containing said variant, may be administered in conjunction with one or more other pharmaceutically active agents. The variant, or the composition containing said variant, may be administered simultaneously (co-administered) with the other treatments or it may be administered sequentially in any order.

The terms "subject" and "individual" are used interchangeably and in the context of the present invention include in their scope any mammal which can develop, or already has, an allergic condition of whatever cause. Of course the preferred subjects for administration of the treatment of the present invention are humans, domestic pets and farm animals.

The required dosage amount will vary according to the severity of the condition to be treated, the cause of the condition, age of the subject and other standard clinical parameters which can be easily determined by routine procedures within the skill-set of those skilled in the art.

It is preferred that *Lactobacillus fermentum* variant VRI 003 or a composition containing it, be administered daily. Of course, it can be administered several times per day, or it may be administered infrequently (for example every second or third day), depending on the progress of treatment of the condition in question, its cause and severity. These parameters can also be easily determined by those skilled in the art.

The dosage form is preferably a tablet or a capsule, but it may also be a powder, liquid or paste/gel.

According to a fourth aspect, the invention provides a method for the prevention or treatment of a gastrointestinal disorder comprising the administration of an effective amount of *Lactobacillus fermentum*, or compositions or components thereof to a subject requiring such treatment.

Preferably the *Lactobacillus fermentum* is the variant VRI 003.

Preferably, the gastrointestinal disorder is irritable bowel syndrome (IBS), inflammatory bowel disease and/or symptoms thereof, such as for example diarrhoea, bloating, flatulence, abdominal cramping, abdominal pain, or constipation. The gastrointestinal disorder may be caused by

pathogenic organisms that may be bacterial, viral or protozoal. However it may also be caused merely by colonisation of the gastrointestinal tract with inappropriate organisms.or by inflammatory and/or autoimmune mechanisms

Preferably, the disturbance or disorder is the result of colonisation of the subject's gastrointestinal tract by a pathogen. Preferably, the disturbance or disorder is the result of a high pathogen load as herein defined. More preferably the pathogen is a bacteria, virus or protozoa. Most preferably, the pathogen is *Salmonella, E. coli, Helicobacter, Vibrio, Pseudomonas, Clostridium*, bacteroides or virus such as Norwalks, rotavirus, or protozoa such as *Cryptosporidium, Entamoeba, Giardia* and *Dientamoeba.*

In the context of the present invention, a "high pathogen load" may occur when a subject has (a) been subjected to attack by a pathogen in an amount not within the normal range of their everyday exposure; (b) been subjected to attack by a virulent pathogen; (c) been subjected to attack by a pathogen at a time when the subject's resistance is lowered, for example, at a time when the immune system is depleted and/or when the subject's other natural defense mechanisms are not functioning normally. The subject's resistance may be low due to, for example, stress or antibiotic treatment.

The *Lactobacillus fermentum* may include live and/or dead cells. Preferably, the *Lactobacillus fermentum* is administered in whole cell live form. However, the administration of components of the *Lactobacillus fermentum* are also contemplated. Components of *Lactobacillus fermentum* include, but are not restricted to, cell fragments, extracts and purified components.

It will be clear to the skilled addressee that the *Lactobacillus fermentum* may be combined with other compounds such as prebiotics, non-digestible dietary components, dietary fibre or pharmaceutically active compounds. Preferably, the prebiotic comprises or consists of inulin, a

resistant starch, an oligosaccharide, a gum or a beta-glucan. Preferably, the prebiotic is an unmodified high amylose maize starch or beta-glucan. The combination of *L. fermentum* and other compound may be in any form eg. a food, liquid, tablet or capsule.

Unless the context clearly requires otherwise, throughout the description and the claims, the words 'comprise', 'comprising', and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 Changes in bacteria in faecal material from IBS subject #4 during an 8 week trial. Microbial profiles monitored during the baseline, placebo, washout and synbiotic treatment periods. Results expressed as colony forming units (cfu) per mL.

Figure 2 Changes in bacteria in faecal material from IBS subject #5 during an 8 week trial. Microbial profiles monitored during the baseline, placebo, washout and synbiotic treatment periods. Results expressed as colony forming units (cfu) per mL.

Figure 3 Changes in bacteria in faecal material from IBS subject #9 during an 8 week trial. Microbial profiles monitored during the baseline, placebo, washout and synbiotic treatment periods. Results expressed as colony forming units (cfu) per mL.

Figure 4 Changes in bacteria in faecal material from IBS subject #2 during an 8 week trial. Microbial profiles monitored during the baseline, placebo, washout and synbiotic treatment periods. Results expressed as colony forming units (cfu) per mL.

Figure 5 Changes in bacteria in faecal material from IBS subject #3 during an 8 week trial. Microbial profiles monitored during the baseline, placebo, washout and synbiotic treatment periods. Results expressed as colony forming units (cfu) per mL.

Figure 6 Changes in bacteria in faecal material from IBS subject #7 during an 8 week trial. Microbial profiles monitored during the baseline, placebo,

washout and synbiotic treatment periods. Results expressed as colony forming units (cfu) per mL.

Figure 7 Changes in bacteria in faecal material from IBS subject #6 during an 8 week trial. Microbial profiles monitored during the baseline, placebo, washout and synbiotic treatment periods. Results expressed as colony forming units (cfu) per mL.

DETAILED DESCRIPTION OF THE INVENTION

Although there have been various attempts to use probiotics in the prevention and treatment of many disorders, the evidence for the use of probiotics in the treatment of gastrointestinal disorders has been very variable. It has now been found that *Lactobacillus fermentum* variant VRI 003, is highly effective in the prevention and/or treatment of gastrointestinal disorders. Specifically, the present invention provides the bacterium *Lactobacillus fermentum* variant VRI 003. It also provides a method for the treatment of a gastrointestinal disorder comprising the administration of an effective amount of *Lactobacillus fermentum* variant VRI 003 or components or compositions thereof to a subject. The *Lactobacillus fermentum* variant VRI 003 can be combined with other components, for example, a prebiotic or non-digestible dietary component.

The methods and compositions of the present invention have been developed for human and veterinary applications in the treatment of gastrointestinal disorders but as a result of the commonality of the mucosal system the treatments may be applied to other disorders of mucosal surfaces and conditions resulting from mucosal surface disturbances or disturbances in the immune system or status of a subject. Whether used for treatment of humans or domestic animals, the underlying principles are the same and advantageously the treatments of the present invention may be used irrespective of the cause of the conditions described above.

Typically the effective daily dosage is in the range of about 10^8-10^{12} bacteria and frequency of administration is once or twice daily. If treating

animals such as dogs and cats, the appropriate dosage can be introduced in food.

Lactobacillus fermentum variant VRI 003 can be formulated by known means, using conventional pharmaceutically acceptable carriers, excipients, solvents or adjuvants. Such procedures and ingredients are well know and amply described in standard texts and manuals, for example "Remington: The Science and Practice of Pharmacy", 1995, Mack Publishing Co. Easton, PA 18042, USA, which is incorporated herein by reference.

Lactobacillus fermentum variant VRI 003 may also be formulated into food products by the usual well-known means.

Preferred embodiments of the invention will now be described by way of example only.

EXAMPLES

Example 1: Origin and Identification

The VRI 003 variant was isolated from a healthy subject. In a series of laboratory experiments, the VRI 003 variant was found to adhere to the gastrointestinal epithelial tissue. It was also shown to have a demonstrable effect on human gastrointestinal pathogens, and was resistant to bile acids. The VRI 003 variant also survived in a low pH environment and was resistant to pepsin and to nutrient limited conditions.

The bacterial variant VRI 003 can be cultured on Rogosa agar (Oxoid) Plates were incubated at 37C in an anaerobic chamber for 24 hours The strain was purified by successive transfers on MRS agar (Oxoid) plates incubated at 37C in an anaerobic chamber for 24 hours and the final culture stored at –70°C in 20% glycerol by subculturing in MRS broth at 37C for 24 hours in anaerobic conditions prior to the addition of glycerol to the culture broth .

Variant VRI 003 was catalase negative, gram positive rod which produced gas when grown anaerobically on brain heart infusion (BHI) (Oxoid) broth containing glucose. It was broadly identified therefore as an heterofermentative lactobacillus and confirmed to be *L. fermentum* strain

according to the API carbohydrate kit (50 CHL kit; Biomerieux; supplier data base and database with a certainty of 99.9%. In particular, sugars 5,10,11,12,13,25,28,29,30,31,32, and 25 are utilized by strain 003. The cell is a short rod, Gram positive and consistent with the description of the morphology of *Lactobacillus fermentum*

Lactobacillus fermentum strain VRI 003 was deposited under the provisions of the Budapest Treaty, at the Australian Government Analytical Laboratories, PO BOX 385 Pymble 2073, NSW, Australia, on 27 August 2002 and the deposit was allocated the accession number NM02/31074.

Example 2: Characteristics and Description of Strain VRI 003

(i) Colony morphology

When grown on MRS (Oxoid) agar in an anaerobic chamber at 37 C, the colonies are approximately 1mm in diameter, shiny, dome shaped, opaque and when touched with a loop show a stickiness with evidence of an extracellular polymer. Incubation of similar plates in aerobic conditions at 37 C yields colonies of rough appearance and irregular edge. Subculturing of these rough colonies onto MRS agar and incubation at 37 C in the anaerobic chamber yields opaque dome shaped, shiny opaque colonies that are sticky to touch.

(ii) Growth in Broth

The culture of variant VRI 003 in MRS (Oxoid) broth, or in various other broths, result is a viscous broth when grown at 37 C in anaerobic conditions.

(iii) Adhesion

While the capacity to colonize the human gastrointestinal tract is not a pre-requisite for the active function of a probiotic strain in the digestive tract, it is a desirable characteristic. If the probiotic strain can adhere to the gastrointestinal tract epithelium, it can colonise, that is establish and grow

within the tract and continually produce metabolites that may mediate the beneficial effect.

The variant VRI 003 colonises the human digestive tract when orally administered to a range of humans. From *in vitro* pathogen inhibition studies it was apparent that the strain produces metabolites that inhibit the growth of a range of potential pathogens, both Gram negative and Gram positive species.

(iv) Antagonistic effects.

Without wishing to be bound by any particular mechanism of action, for a probiotic strain to effectively protect the subject from pathogens of the gastrointestinal tract, it may need to produce some metabolites inhibitory to the growth of the pathogen. This inhibitory effect on growth is referred to as an antagonistic effect and the antagonistic metabolites can be classified as low or high molecular weight compounds. Three different methods have been used to evaluate the antagonistic capacity of strain VRI 003 against a range of human gastrointestinal pathogens.

The most convenient method for screening a large number of pathogens is to use point inocula of the *Lactobacillus* that are overlaid with the pathogens and the size of the zone of growth inhibition of the pathogen measured after incubation. To quantify further the antagonistic effect detected using this method, the VRI 003 was co-cultured with the pathogen in liquid medium and the number of viable pathogen cells enumerated after 24 hours.

The inhibitory activity of strain VRI 003 was also examined to determine whether low and high molecular weight metabolites of the lactobacillus mediated the growth inhibitory effects detected. The bacterial spent culture fluid of VRI 003 was collected and one part dialysed to retain only compounds with a molecular weight greater than 8,000. The growth of selected human pathogens was studied in the absence and presence of the dialysis retentates and the non-dialysed spent culture fluids.

From *in vitro* pathogen inhibition studies it was apparent that the strain produces metabolites that inhibit the growth of a range of potential pathogens, both Gram negative and Gram positive species. The variant VRI 003 produced both low and high molecular metabolites that could inhibit pathogens and protect in animal challenge studies.

Example 3: Culture and Formulations

(i) Growth of the culture

Lactobacillus fermentum variant VRI 003 is grown in a fermentation vessel at 37°C. The vessel is then cooled and the fermentation broth concentrated, preferably by centrifugation. The collected culture is dried, preferably by freeze-drying and subsequently milled. The milled material is then blended with the major excipient to give the desired level of microbes per gram of dry material. The level to be used is dependant on the application (range up to log 11 per gram). The standardised material is then used in the formulation by mixing all ingredients in a blender (preferably a V-blender).

(ii) Formulations

(a) Formulation A: High amylase maize based (symbiotic formulation)

Lactobacillus fermentum VRI 003	100mg
Hi-maize 958 (or 1043)	170mg
Stearic acid	up to 4.5mg
Silica dioxide	up to 4.5mg

(b) Formulation B: Microcrystalline cellulose (MCC) based

Lactoabcillus fermentum VRI 003	100mg
Avicell 112 (or equivalent)	170mg
Stearic acid	up to 4.5 mg
Silica dioxide	up to 4.5 mg

(c) Formulation C: Either the High amylase maize base or the MCC based as described in A and B, with colloidal silica (up to 4.5 mg) instead of silica dioxide or with silica dioxide as well (up to 4.5 mg).

One of the desired characteristics for VRI 003 is that it remains viable and has the capacity to grow within the human gastrointestinal tract after dosage. As outlined above this characteristic was one of the selection criteria for a desirable strain. However, this is not necessarily essential for the desired beneficial effects.

An additional important factor in this regard is that even though the strain has the capacity to survive the various conditions within the tract, the strain must retain viability and desired strain characteristics when grown on a large scale and dispensed in a product form. All results presented above were based on VRI 003 cells harvested directly from actively growing laboratory cultures. The following is an analysis of the viability and strain characteristics of VRI 003 after large scale culture and freeze-drying, as well as after encapsulation in gelatin capsules.

(iii) Viability of Freeze-Dried VRI 003.

The viability of VRI 003 after large scale production and freeze-drying was determined by analysing the colony forming units per gram (CFU/ g) of dried material. The dried powder was examined both before and after encapsulation in the gelatin capsules. The number of viable cells was determined for ten individual 1g samples of the powder. The contents of ten capsules were also individually analysed.

VRI 003 maintained high levels of viability through production and encapsulation. The dried powder contained 5.6×10^{10} cfu/g and the contents of the capsules contained 4.15×10^{10} CFU/g; results expressed as the mean where n = 10.

When the capsules were stored in foil/foil packaging, a 1.5 log, or less, loss of viability was noted with storage at 30C and 25C for 6 months.

(iv) Variant characteristics

The dried powder and capsule contents were suspended in phosphate buffered saline (0.1M, pH 7.2) to yield a 100-fold dilution. The variant characteristics outlined above were tested on this bacterial suspension. For all tests, an actively growing culture of VRI 003 was included as the internal control. As detailed in the following no marked loss of strain characteristics was noted for the freeze-dried powder or for the capsule contents.

Further, the powder and capsule contents showed similar adhesion characteristics to those of laboratory grown control culture.

Example 4: Effect of administration of Lactobacillus fermentum VRI 003, with or without a prebiotic, on symptoms of irritable bowel syndrome (IBS) and gastrointestinal flora profile

(i) Experimental design

Participants

Seven patients with IBS (2 male and 5 female) participated in the study after giving written informed consent (HREC 99264). All patients had not taken antibiotics 3 months prior to commencement of the study. Patients were recruited by public advertising in the local newspaper and also from the Sydney Clinic for Gastrointestinal Diseases at Bondi Junction, Sydney, Australia.

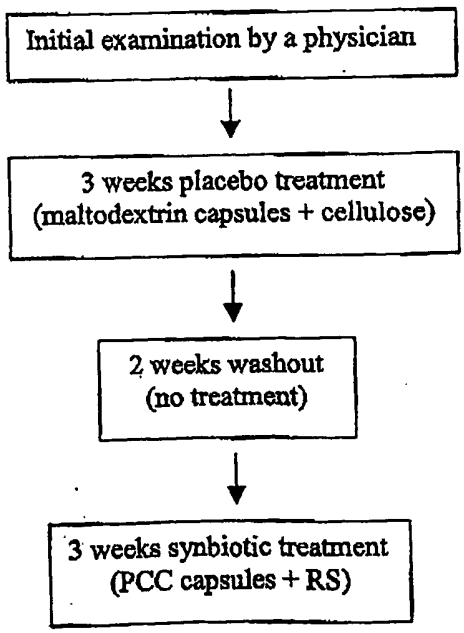
All patients were examined by a physician prior to participation in the trial for presentation of IBS diagnostic criteria and absence of organic disease. All patients were symptomatic at the time of the study.

Trial design

The single-blinded placebo trial extended for a duration of 8 weeks, consisting of 3 weeks placebo treatment followed by two weeks washout (no treatment) and concluding with 3 weeks synbiotic treatment, ie. treatment with a formulation containing VRI 003 variant and a prebiotic. A smaller group of patients was treated with a formulation containing VRI 003 alone.

Patients' diets were not controlled in the study, however they were advised to refrain from consuming fermented milk products (e.g. yoghurt and sour cream). Fresh faecal samples were requested from the patients on day one of the trial to form the baseline, and then on days 21, 35 and 56 (before and after each treatment period). An initial questionnaire was completed by the patients on the first day of the trial and thereafter weekly questionnaires were completed by the patients for each week of the trial.

Typical treatment protocol was as follows (provided for the symbiotic formulation but a similar protocol was used for a formulation with VRI 003 alone)



Capsules and carbohydrates

Empty capsules were manually filled. Assembly of capsules was performed in a laminar flow hood and materials were sterilized for 15 min under UV prior to use. Placebo capsules contained microcrystalline cellulose NF XVIII (Mingtai Chemical Company Ltd, Taiwan), while probiotic capsules consisted of *Lactobacillus fermentum* (VRI 003) as a freeze-dried powder ($1.67X10^{10}$ cfu.gm^{-1}, DSM Moorebank, Sydney). Assembled capsules were placed into 120 mL specimen jars with two sachets of silica gel and stored in a refrigerator designated for food materials for human consumption.

Maltodextrin (Fieldstar™, Goodman Fielder, Sydney) and resistant starch (Culture Pro™ 958, Penfold Australia) were utilized as the placebo and prebiotic carbohydrates respectively. Carbohydrates were placed under UV for 15 min in a laminar flow hood, before samples were weighed out (20 g) and placed into 70 mL specimen jars which were stored in the clean room.

Questionnaires

An initial questionnaire was used to establish the patients' previous and present symptoms prior to commencement of the trial. Patients were asked to rate the severity of their symptoms, including diarrhoea, constipation, alternating diarrhoea and constipation, flatulence, bloating and crampy abdominal pain, on a scale of 0 (absent) through to 10 (extreme). Other questions relating to causes and relief of gut disturbances and food allergies were also queried.

Patients were required to complete a weekly questionnaire at the end of each week for the duration of the trial which again asked them to rate the severity of their symptoms and also the nature of any gut disturbances. In addition, questions regarding the consumption and apparent effects of the capsules and carbohydrate were also queried.

Preparation of faecal material

Faecal inoculum was collected from subjects with IBS whom had not taken antibiotics for three months prior to commencement of the trial. Faeces were collected into a clean plastic container and transferred into the anaerobic chamber upon arrival. One part faeces was homogenized with three parts half-strength Wilkin-Chalgrens (WC) broth (Oxoid, CM643) in a Seward stomacher bag.

Enumeration of bacteria

A ten-fold serial dilution was performed in half-strength Wilkin-Chalgrens (WC) broth (Oxoid, CM643) in sterile microcentrifuge tubes, after which 10 µL aliquots were drop-plated in triplicate onto various media (table 1), in order to assess the numbers of major microbial groups present in the faecal samples. Media were prepared according to the manufacturer's recommendations. Samples plated onto RCA plates were first heated to 90°C for 10 min.

Table 1 Bacteria populations enumerated in fermentation.

Medium	Microorganisms	Dilutions plated	Incubation
Nutrient Agar (NA; CM3*)	Total aerobes	10^{-4} to 10^{-8}	O_2, 24 hr
MacConkey Agar (MAC; CM7*)	Enterobacteria	10^{-4} to 10^{-8}	O_2, 24 hr
WC + Blood[1] Agar (CM619*)	Total anaerobes	10^{-4} to 10^{-8}	AnO_2, 48 hr
WC + Blood[1] + Antibiotic supplement[2] (CM619*)	Gram negative anaerobes	10^{-4} to 10^{-8}	AnO_2, 48hr
Rogosa Agar (CM627*)	Lactobacilli	10^{-4} to 10^{-8}	AnO_2, 48 hr
Raffinose Bifibacterium Agar (RB; Hartemink, 1995)	Bifidobacteria	10^{-4} to 10^{-8}	AnO_2, 48hr

Reinforced Clostridial Agar (RCA; CM151*)	Clostridia	10^{-1} to 10^{-5}	AnO_2, 72hr

* Oxoid

[1] Oxoid defibrinated blood (50 mL per 1 L agar)

[2] Oxoid G-N anaerobe selective supplement SR108B

(ii) Effects of administration of L fermentum VRI 003 alone or in combination with a prebiotic - Summary results

Table 2: Effects of L fermentum strain VRI 003 on the severity of symptoms of IBS when administered alone or together with a prebiotic (high amylose maize starch) (resistant starch = RS). Results expressed as "-" which corresponds to no symptoms to "+" up to "+++" where the more severe symptoms are presented as "+++".

Symptoms	Baseline	Placebo	VRI 003+RS	VRI 003 alone
Diarrhoea	+++	+++	+	+
Bloating	+++	++	+	++

Administration of VRI 003 was effective even when administered alone. However, the combination of VRI 003 with beta-glucan elevated the levels of VRI 003 bacteria and decreased levels of certain bacteria such as *Salmonella* and *Clostridium* when compared to the probiotic alone or the beta-glucan alone.

Example 5: Changes in the faecal microbial profiles using material from IBS subjects during the trial

Results have been obtained for changes in the total aerobes, enteric bacteria, total anaerobes, Gram negative anaerobes, lactobacilli, clostridia and bifidobacteria during the trial. Time points displayed on the graph are representative of the faecal microbal profile at the start of the trial (baseline), end of placebo treatment, end of washout period and end of

synbiotic treatment. In regard to the results, subjects were separated into the subgroups, diarrhoea-, constipation- and alternating diarrhoea and constipation predominant, according to the symptoms they normally experience with the condition. This subgrouping allows subjects to be appropriately assessed due to the wide variability in symptoms experienced by those with the condition (Akehurst and Kaltenthaler, 2001). One subject, however, did not fit into either of the three subgroups and has been placed into a separate group for flatulence, bloating and crampy abdominal pain predominant.

Diarrhoea predominant subjects

Changes in the faecal microbial profile of diarrhoea predominant subjects are shown in figure 1 (subject 4), figure 2 (subject 5) and figure 3 (subject 9).

Subject 4 (figure 1): Total aerobes remained at a constant level of $1x10^5$ log cfu.mL^{-1}, except during the washout period, in which total aerobes increased by one log. Numbers of enteric bacteria which were initially detected at $1x10^5$ log cfu.mL^{-1} decreased by one and a half log by the end of the synbiotic treatment period, though enteric bacteria began to display a widening disparity compared to numbers of total aerobes at the end of the washout period. Both total anaerobes and Gram negative anaerobes, which began at $5x10^8$ log cfu.mL^{-1}, displayed an increase of one log by the end of the synbiotic treatment period. Lactobacilli were initially detected at $1x10^7$ log cfu.mL^{-1} and exhibited a decrease of two log at the end of the placebo and synbiotic treatment periods, which peaked again in between, during the washout period. Clostridia remained undetectable throughout the whole trial, while numbers of bifidobacteria, which began at $5x10^7$ log cfu.mL^{-1}, increased by one log during the synbiotic treatment period.

Subject 5 (figure 2): Total aerobes, which were initially detected at $5x10^5$ log cfu.mL^{-1}, increased by one log during the washout and synbiotic treatment periods. Enteric bacteria remained at levels similar to those of the total aerobes ($5x10^5$ log cfu.mL^{-1}) during all time points, except at the end of the placebo treatment period, in which numbers of enteric bacteria decreased by two log. Numbers of total anaerobes and Gram negative

anaerobes continued throughout the trial at a constant level of $1x10^9$ log cfu.mL^{-1}, though decreased by one to one and a half log at the end of the placebo treatment period. Lactobacilli, which began at $1x10^6$ log cfu.mL^{-1}, decreased by one log at the end of the placebo treatment period but increased by three log at the end of the washout period, only to decrease again by one log at the end of the synbiotic treatment period. Clostridia was detected at the beginning of the trial at $1x10^3$ log cfu.mL^{-1} and remained undetectable throughout the rest of the trial. Bifidobacteria, which began at $5x10^6$ log cfu.mL^{-1}, decreased by one log at the end of the placebo and synbiotic treatment period, but increased by one log in between during the washout period.

Subject 9 (figure 3): Total aerobes and enteric bacteria were detected at $1x10^7$ log cfu.mL^{-1} at the baseline and proceeded to decrease slowly by one and a half log by the end of the synbiotic treatment period. Total anaerobes and gram negative anaerobes were detected at $5x10^8$ log cfu.mL^{-1} at the baseline, increased by one log at the end of the placebo treatment period and remained at a steady level for the rest of the study. Lactobacilli were detected at $5x10^5$ log cfu.mL^{-1} at the baseline, however became undetectable during the placebo treatment period. Lactobacilli were detected again at $5x10^3$ log cfu.mL^{-1} at the end of the washout period and increased by one log at the end of the symbiotic treatment period. Clostridia were absent for the duration of the trial. Bifidobacteria began at $5x10^7$ log cfu.mL^{-1}, decreased by one a half log at the end of the placebo treatment period, however increased again to $5x10^8$ log cfu.mL^{-1} by the end of the washout period and remained at this level till the end of the synbiotic treatment period.

Constipation predominant subjects

Changes in the faecal microbial profile of constipation predominant subjects are shown in figure 4 (subject 2) and figure 5 (subject 3).

Subject 2 (figure 4): Total aerobes and enteric bacteria were initially detected at $1x10^7$ log cfu.mL^{-1} and remained at similar levels throughout the trial, except at the end of the placebo treatment period, where numbers of enteric bacteria increased by one log. Numbers of total anaerobes and Gram negative anaerobes remained at similar levels of $1x10^8$ log cfu.mL^{-1}

throughout the trial, with an increase of one log at the end of the placebo treatment period, which decreased by half a log by the end of the synbiotic treatment period. Lactobacilli, which began at $1x10^3$ log cfu.mL^{-1}, increased by one log at the end of the placebo treatment period, but became undetectable at the end of washout period and increased again by three log by the end of the synbiotic treatment period. Clostridia was present only at the beginning of the trial at $1x10^3$ log cfu.mL^{-1} and remained undetectable throughout the rest of the trial. Bifidobacteria were initially detected at $1x10^7$ log cfu.mL^{-1} and decreased by one and a half log at the end of the placebo treatment period, and then became undetectable during the rest of the trial.

Subject 3 (figure 5): Total aerobes and enteric bacteria remained at similar levels of $5x10^4$ log cfu.mL^{-1} for the duration of the trial and increased by two log at the end of the placebo treatment period, though decreased by one log by the end of the synbiotic treatment period. Total anaerobes and Gram negative anaerobes began at $1x10^7$ log cfu.mL^{-1} and continued a steady increase of two log by the end of the synbiotic treatment period. Lactobacilli were detected at the beginning of the trial at $1x10^4$ log cfu.mL^{-1}, but then became undetectable at the end of the placebo treatment period. However, lactobacilli were shown to increase by four and a half log at the end of the washout period and decrease again by half a log at the end of the synbiotic treatment period. Clostridia were only detected at the end of the placebo treatment period at $1x10^4$ log cfu.mL^{-1} and remained undetectable for the rest of the trial. Bifidobacteria, which began at $1x10^7$ log cfu.mL^{-1} was not able to be detected at the end of the placebo treatment period, though became detectable again by the end of the washout period and increased by four and a half log by the end of the synbiotic treatment period.

Alternating diarrhoea and constipation predominant subject

Changes in the faecal microbial profile of an alternating diarrhoea and constipation predominant subject is shown in figure 6 (subject 7).

Subject 7 (figure 6): Total aerobes and enteric bacteria which were initially detected at $5x10^5$ log cfu.mL^{-1}, increased by two log at the end of

the washout period, however decreased by one log at the end of the synbiotic treatment period. Total anaerobes and Gram negative anaerobes began at $5x10^8$ log cfu.mL^{-1} and remained at a steady level throughout the study, until the synbiotic treatment period, where total anaerobes decreased by one log while Gram negative anaerobes decreased by two log. Lactobacilli were not detected for most part of the study, except by the end of the synbiotic treatment period, at $5x10^3$ log cfu.mL^{-1}. In contrast, clostridia was only detected at the beginning of the trial at $1x10^3$ log cfu.mL^{-1}. Bifidobacteria were initially detected at $5x10^6$ log cfu.mL^{-1}, however became undetectable during the placebo treatment period, only to be detected again during the washout period at $5x10^7$ log cfu.mL^{-1} and increased by half a log at the end of the synbiotic treatment period.

Other: flatulence, bloating and crampy abdominal pain predominant subject

Changes in the faecal microbial profile of a flatulence, bloating and crampy abdominal pain predominant subject is shown in figure 7 (subject 6).

Subject 6 (figure 7): Total aerobes and enteric bacteria were initially detected at $5x10^5$ log cfu.mL^{-1} and remained level until the synbiotic treatment period, which saw a two log increase in total aerobes and enteric bacteria. Total anaerobes and Gram negative anaerobes were detected at $1x10^8$ log cfu.mL^{-1} at the baseline and increased by one log at the end of the placebo treatment period, after which they remained at this level for the rest of the study. Lactobacilli remained at a relatively stable level of $1x10^4$ log cfu.mL^{-1} for the duration of the study. Clostridia was not detected during the study, except at the end of the synbiotic treatment period at $5x10^3$ log cfu.mL^{-1}. Bifidobacteria began at $1x10^7$ log cfu.mL^{-1} and decreased by three log by the end of the placebo treatment period, only to increase again to $1x10^7$ log cfu.mL^{-1} during the washout period and finally decreased by three log at the end of the synbiotic treatment period.

Example 6: Changes in the severity of symptoms of IBS subjects during the trial

Results have been obtained for the changes in the severity of symptoms including diarrhoea, constipation, alternating diarrhoea and constipation, flatulence, bloating and crampy abdominal pain. Each symptom is graded on a scale of 0 to 10, with 0 indicating absence of the symptom and 10 indicating extreme severity of the symptom. Results are displayed on a scale of 1 to 5, with 1 indicating mild severity of the symptom and 5 indicating extreme severity of the symptom. Changes in the severity of symptoms were monitored every week in all subjects, however, the results will display only time points representing the baseline (beginning of the trial), placebo treatment period, washout period and synbiotic treatment period. Subjects were again separated into subgroups, diarrhoea-, constipation- and alternating diarrhoea and constipation predominant in accordance with the symptoms they normally experience with the condition.

Diarrhoea predominant subjects

Changes in the severity of symptoms for diarrhoea predominant subjects are shown in table 3 (subject 4), table 4 (subject 5) and table 5 (subject 9).

Subject 4 (table 3): The severity of diarrhoea at the baseline was extremely high, however this was reduced to half the severity by the end of the placebo treatment period, which further decreased down to low severity by the end of the synbiotic treatment period. Flatulence and bloating were at medium severity (3) at the baseline and eventually reduced to low severity (1) by the end of the synbiotic treatment period. Crampy abdominal pain was also low (2) at the start of the baseline and further reduced in severity down to 1 by the end of the synbiotic treatment period.

Table 3 Changes in the severity of symptoms of subject #4 during an 8 week trial. Severity of symptoms were monitored during the baseline,

Symptom	Baseline	Placebo	Washout	Synbiotic
Diarrhoea	+++++	++	++	+
Constipation	-	-	-	-
Flatulence	+++	+++	++	+
Bloating	+++	++	++	+
Crampy abdominal pain	++	++	++	+

placebo, washout and synbiotic treatment periods. Results expressed as + (low severity) to + + + + + (extreme severity).

Subject 5 (table 4): The severity of diarrhoea at the baseline was extremely high and only decreased slightly to 4 by the placebo treatment period, after which it remained at high severity. Flatulence was low at the baseline, absent during the placebo treatment period, but increased again during the washout and synbiotic treatment periods by 1 and 2 respectively. The severity of crampy abdominal pain remained at the same level of 3 throughout the trial, though decreased a little by 1 during the washout period. Symptoms of flatulence and bloating remained absent throughout the duration of the trial.

Table 4 Changes in the severity of symptoms of subject #5 during an 8 week trial. Severity of symptoms were monitored during the baseline, placebo, washout and synbiotic treatment periods. Results expressed as + (low severity) to + + + + + (extreme severity).

Symptom	Baseline	Placebo	Washout	Synbiotic
Diarrhoea	+++++	++++	++++	++++
Constipation	-	-	-	-
Flatulence	+	-	+	++
Bloating	-	-	-	-
Crampy abdominal pain	+++	+++	++	+++

Subject 9 (table 5): Diarrhoea began at mid range (3) at the baseline and continued at this severity up until the synbiotic treatment period, where it had reduced by 2 down to low severity. Constipation was absent during the trial, apart from the placebo treatment period, in which it had increased by 1. Flatulence was also absent during the trial, however increased by 3 during the washout period. Bloating, which was at medium severity (3) at the baseline, reduced by 1 during the placebo treatment period. However, this increased again by 1 during the washout period and then dropped

down to low severity (1) during the probiotic treatment period. Crampy abdominal pain was absent throughout the duration of the trial.

Table 5 Changes in the severity of symptoms of subject #9 during an 8 week trial. Severity of symptoms were monitored during the baseline, placebo, washout and synbiotic treatment periods. Results expressed as + (low severity) to + + + + + (extreme severity).

Symptom	Baseline	Placebo	Washout	Synbiotic
Diarrhoea	+++	+++	+++	+
Constipation	-	+	-	-
Flatulence	-	-	+++	-
Bloating	+++	++	+++	+
Crampy abdominal pain	-	-	-	-

Constipation predominant subjects

Changes in the severity of symptoms for constipation predominant subjects are shown in table 6 (subject 2) and table 7 (subject 3).

Subject 2 (table 6): Diarrhoea, which was absent at the baseline and for the most part of the trial, increased by 1 during the synbiotic treatment period. Constipation, flatulence and bloating remained at high severity (5) at the baseline and during the placebo and washout periods, though decreased by 1 during the synbiotic treatment period. Crampy abdominal pain was at high severity (4) at the baseline and during the placebo treatment period, however reduced by 3 down to low severity during the washout and synbiotic treatment period.

Table 6 Changes in the severity of symptoms of subject #2 during an 8 week trial. Severity of symptoms were monitored during the baseline,

Symptom	Baseline	Placebo	Washout	Synbiotic
Diarrhoea	-	-	-	+
Constipation	+++++	+++++	+++++	++++
Flatulence	+++++	+++++	+++++	++++
Bloating	+++++	+++++	+++++	++++
Crampy abdominal pain	++++	++++	+	+

placebo, washout and synbiotic treatment periods. Results expressed as + (low severity) to + + + + + (extreme severity).

Subject 3 (table 7): Constipation began at low severity (2) at the baseline and continued at this level during the placebo treatment period, however became absent during the washout and synbiotic treatment periods. Flatulence began at very high severity (5) at the baseline, reduced down to 3 during the placebo treatment period and further reduced down to low severity (2) during the washout and synbiotic treatment periods. Bloating also began at very high severity at the baseline and was also able to reduce in severity during the placebo, washout and synbiotic treatment periods to 4, 3 and 3 respectively. Crampy abdominal pain began at high severity (4) at the baseline and reduced to low severity (1) during the placebo treatment period, after which it became absent for the remainder of the trial. Diarrhoea was absent for the duration of the trial.

Table 7 Changes in the severity of symptoms of subject #3 during an 8 week trial. Severity of symptoms were monitored during the baseline, placebo, washout and synbiotic treatment periods. Results expressed as + (low severity) to + + + + + (extreme severity).

Symptom	Baseline	Placebo	Washout	Synbiotic
Diarrhoea	-	-	-	-
Constipation	++	++	-	-
Flatulence	+++++	+++	++	++
Bloating	+++++	++++	+++	+++
Crampy abdominal pain	++++	+	-	-

Alternating diarrhoea and constipation predominant subject

Changes in the severity of symptoms for an alternating diarrhoea and constipation predominant subject is shown in table 8 (subject 7).

Subject 7 (table 8): Diarrhoea started at low severity (1) at the baseline but increased to 2 during the washout and synbiotic treatment periods. Constipation, which was at low severity (2) at the baseline, decreased by 1 during the placebo and washout periods, but increased to 3 during the synbiotic treatment period. Flatulence began at low severity (1) at the baseline and remained low for the most part of the trial, however it increased to 3 during the synbiotic treatment period. Bloating began at low severity (2) and decreased to 1 during the placebo treatment period, however continued to increase during the washout and synbiotic treatment periods to 3 and 4 respectively. Crampy abdominal pain was absent during the trial, except during the synbiotic treatment period, when it had increased to medium severity (3).

Table 8 Changes in the severity of symptoms of subject #7 during an 8 week trial. Severity of symptoms were monitored during the baseline, placebo, washout and synbiotic treatment periods. Results expressed as + (low severity) to + + + + + (extreme severity).

Symptom	Baseline	Placebo	Washout	Synbiotic
Diarrhoea	+	+	++	++
Constipation	++	+	+	+++
Flatulence	+	+	+	+++
Bloating	++	+	+++	++++
Crampy abdominal pain	-	-	-	+++

Other: flatulence, bloating and crampy abdominal pain predominant subject

Changes in the severity of symptoms for a flatulence, bloating and crampy abdominal pain predominant subject is shown in table 9 (subject 6).

Subject 6 (table 9): Diarrhoea was absent for most of the trial, except during the synbiotic treatment period, in which it had increased by 1 to low severity. Flatulence began at medium severity (3) at the baseline and reduced to low severity (1) during the placebo and washout periods, however increased up to medium severity (3) once again during the

synbiotic treatment period. Bloating also began at medium severity (3) at the baseline and became absent during the placebo treatment period, however it had also increased again during the washout and synbiotic treatment periods to 1 and 3 respectively. Crampy abdominal pain began at low severity (2) at the baseline, was absent during the placebo treatment period but increased by 1 and 3 during the washout and synbiotic treatment periods respectively. Constipation remained absent for the duration of the trial.

Table 9 Changes in the severity of symptoms of subject #6 during an 8 week trial. Severity of symptoms were monitored during the baseline, placebo, washout and synbiotic treatment periods. Results expressed as + (low severity) to + + + + + (extreme severity).

Symptom	Baseline	Placebo	Washout	Synbiotic
Diarrhoea	-	-	-	+
Constipation	-	-	-	-
Flatulence	+++	+	+	+++
Bloating	+++	-	+	+++
Crampy abdominal pain	++	-	+	+++

Discussion

Correlations between changes in the faecal microbial profiles using material from IBS subjects and severity of symptoms during the trial

The clinical trial lasted for a total duration of 8 weeks, the first 3 weeks involved a placebo treatment consisting of maltodextrin capsules and cellulose. This was followed by a 2 week washout period in which no treatment was taken. The last 3 weeks involved a symbiotic treatment consisting of VRI 003 capsules and RS. Faecal samples were collected at the beginning of the trial to establish a baseline, after which further samples were taken before and after each treatment period. In addition, weekly questionnaires were also completed by each patient to monitor changes in their severity of symptoms via a scoring system graded from 0 to 10, with 0 being the absence of the symptom and 10 being the most

severe. Results were then converted to a scale of 1 to 5 for convenience and shown for the baseline, placebo, washout and symbiotic treatment periods.

Patients were grouped into 3 subgroups according to their predominance in symptoms, as shown by Akehurst *et al* and Weston *et al* (Akehurst and Kaltenthaler, 2001; Weston, et al., 1993). Separating patients into either diarrhoea, constipation or alternating diarrhoea and constipation predominant subgroups allows changes in particular symptoms to be assessed accordingly, due to wide variability among different patients in the symptoms they normally experience with the condition. One subject in particular (#6) displayed symptoms unlike those experienced by any of the other patients in the 3 subgroups, and was thereby grouped into an others group for flatulence, bloating and crampy abdominal pain predominant symptoms.

Diarrhoea predominant subjects

Subject 4 exhibited a general decrease in the severity of all symptoms (table 3) by the end of the synbiotic treatment period, in particular diarrhoea, which started off at high severity at the baseline and then proceeded to low severity by the end of the synbiotic treatment period. A possible correlation with this symptom may be seen in the slow decrease of enteric bacteria (figure 1) from $1x10^5$ log cfu.mL^{-1} at the baseline, down to $5x10^3$ log cfu.mL^{-1} at the end of the synbiotic treatment period. Numbers of lactobacilli do not seem to correlate with any of the symptoms displayed by subject 4, however bifidobacteria were observed to increase by one log during the synbiotic treatment period, which correlates with previous discussion that bifidobacteria are able to utilize RS. However, it is unlikely that the increase in bifidobacteria resulted in the decrease of diarrhoea, as reduction in the severity of this symptom began during the placebo period, so it may be that in this subject, enteric bacteria may have had more of an effect on the severity of diarrhoea.

Subject 5 did not appear to display an overall reduction in the severity of any symptoms (table 4) during the trial. However, it may be noted that during the placebo treatment period, absence of flatulence was observed, which may be related to a two log decrease in the numbers of enteric bacteria (figure 2). The general overview of this subject would indicate that the synbiotic treatment did not appear to promote any beneficial effects on the individual, but rather the placebo treatment may have had some beneficial effect on the severity of symptoms. Numbers of lactobacilli increased by three log during the washout period, though decreased one log by the end of the synbiotic treatment period. The large increase in lactobacilli during the washout period may also relate to the decrease in crampy abdominal pain exhibited by the subject during this time, which then increased in severity by the end of the synbiotic treatment period, along with a minor decrease in lactobacilli during this period. The observation of an increase in lactobacilli associated with a decrease in crampy abdominal pain may also relate to the finding by Nobaek *et al*, which observed that administration of *Lactobacillus plantarum* decreased pain and flatulence in patients with IBS (Nobaek, et al., 2000). A one log increase in bifidobacteria was also observed during the washout period, which also correlates with an increase in lactobacilli, as bifidobacteria are hypothesized to indirectly promote the growth of lactobacilli. Although symptoms did not appear to improve during the synbiotic treatment period in this subject, comments in the weekly questionnaires indicate that symptoms stayed the same during the treatment, rather than increase in severity.

Subject 9 exhibited an overall improvement in the severity of all symptoms (table 5) by the end of the trial, particularly diarrhoea and bloating, which had reduced from medium severity down to low severity by the end of the synbiotic treatment period. The decrease in these symptoms may be related to a two log decrease in enteric bacteria from the baseline to the end of the synbiotic treatment period. Bifidobacteria were also observed to increase by three log after the placebo treatment period. However, this increase began during the washout period, when symptoms still remained

at medium severity so numbers of bifidobacteria may not have had an effect on the lowering of symptoms during the synbiotic treatment period. Numbers of lactobacilli were also observed to increase (one log) by the end of the synbiotic treatment period, which may be correlated with the decrease in severity of symptoms. The increase in bifidobacteria and lactobacilli may also be associated with the administration of RS, which has been shown to promote the growth bifidobacteria and thereby lactobacilli (Brown, et al., 2000).

Constipation predominant subjects

Subject 2 did not appear to display an improvement in most symptoms (table 6), apart from crampy abdominal pain, which greatly decreased in severity during the washout and synbiotic treatment period. Results from the faecal microbial profile (figure 4) show that during the synbiotic treatment period, bifidobacteria were unable to be detected and a decrease (one log) in total aerobes and enteric bacteria were observed during this time. This would indicate that the decrease in enteric bacteria may reduce symptoms of crampy abdominal pain, however it can only be speculated that the absence of bifidobacteria may be related also to a decrease in the symptom, as bifidobacteria are perceived to be beneficial in the human gut. The faecal microbial profile of subject 2 also shows that lactobacilli were not promoted to higher numbers during the synbiotic treatment period. This may be due to low starting numbers of the lactobacilli in the gut of the subject, which may require a longer time period to stimulate to higher numbers. Although the subject did show an overall improvement in symptoms, the weekly questionnaires indicate that symptoms that were experienced during the trial remained steady and did not increase in severity.

Subject 3 displayed an overall general improvement in the severity of symptoms (table 7), particularly constipation and crampy abdominal pain, which subsided by the end of the washout and synbiotic treatment period.

The improvement in these symptoms may be correlated with the large increase (four log) in the number of bifidobacteria (figure 5) at the end of the synbiotic treatment period. The increase in bifidobacteria indicates that the administration of RS during the synbiotic treatment period has stimulated the growth of bifidobacteria. Administration of dietary fibre (e.g. RS) has also been shown to reduce symptoms of constipation through faecal bulking (Lambert, et al., 1991; Phillips, et al., 1995). Although lactobacilli numbers remained low compared to the bifidobacteria during the trial, it is possible that if the trial extended for a longer period of time, lactobacilli may increase in numbers to a larger extent. Enteric bacteria also decreased (one log) by the end of the synbiotic treatment period and though only a minor decrease, it may have also contributed to the decrease in the severity of these symptoms.

Alternating diarrhoea and constipation predominant subject

Subject 7 displays improvement in all symptoms (table 6) during the placebo treatment period, whilst exhibiting an increase in the severity of symptoms during the synbiotic treatment period. A gradual increase in the numbers of enteric bacteria (figure 6) were observed during the trial, which suggests that an increase in enteric bacteria may be related to an increase in symptoms such as bloating and diarrhoea. An important note to be considered however, is that administration of prebiotics, such as RS, are likely to increase symptoms of bloating and flatulence, even in healthy subjects (Cummings *et al.*, 2001; Munster *et al.*, 1994), so an increase in these symptoms experienced by an IBS subject is not unlikely. Bifidobacteria were also observed to increase in numbers during the washout period, while not being able to detected during the placebo treatment period, implying that an increase in bifidobacteria may perhaps be related to an increase in severity of these symptoms. Bifidobacteria were also observed to be detected in very high numbers on RB media and it has been noted that other species of bacteria apart from bifidobacteria may be detected on these plates, such as lactobacilli species (Hartemink *et al.*, 1996). Thus biochemical tests may have to be performed on the colonies growing on RB media to confirm that they were all bifidobacteria

species. Although symptoms appeared to increase in severity during the synbiotic treatment period, comments from the weekly questionnaire indicate that experience of these symptoms were normal in relation to the baseline.

Other: flatulence, bloating and crampy abdominal pain predominant subject

Changes in the severity of symptoms observed for subject 6 (table 9), show that this subject experienced a decrease in the severity of symptoms during the placebo treatment period, while the synbiotic treatment period appeared to have an adverse effect on symptoms. Numbers of bifidobacteria, and to a lesser extent, lactobacilli, were observed to decrease (figure 7) during the placebo treatment period. Bifidobacteria decreased in numbers by three log during the placebo treatment period, corresponding with a decrease in flatulence and absence of bloating and crampy abdominal pain. It can only be suggested however that bifidobacteria may be related to an increase in these symptoms as they are viewed to be beneficial bacteria. It should also be taken into account that administration of prebiotics, particularly RS, has been shown to increase symptoms of bloating and flatulence (Cummings, et al., 2001; Munster, et al., 1994), which were symptoms displayed by subject 6 during administration of RS during the synbiotic treatment period. A general improvement in the severity of symptoms during the placebo treatment period may also be attributed to the possible bulking effect caused by cellulose and thereby ease of defaecation. Weekly questionnaires also indicate that the subject consumed a small amount of alcohol during the washout period, which may have aggravated an increase in symptoms. Furthermore, it was noted this subject experienced stress during the synbiotic treatment period, which may have also increased severity of symptoms, as observed by others (Lidbeck and Nord, 1994; Longstreth and Wolde-Tsadik, 1993).

Trial results of the faecal microbial profiles indicate a general decrease in the number of enteric bacteria correlated with a decrease in the severity of symptoms. An increase in the number of lactobacilli has also been associated with an improvement in some symptoms, such as a reduction in flatulence (subject #5). However, lactobacilli do not seem to exert a very prominent effect in terms of an improvement in symptoms. In comparison, increases in bifidobacteria may appear to have some correlation with symptom severity, though it may be a mixed one. An increase in bifidobacteria may be observed to be related to a decrease in constipation and crampy abdominal pain (subject #3), however increases in bifidobacteria may also be associated with an increase in flatulence and crampy abdominal pain (subject #7). Perhaps different species of bifidobacteria may be involved in increases or decreases in symptom severity, or it may be that different patients are affected by colonization of different gut microorganisms which affect the levels of bifidobacteria in the gastrointestinal tract.

The trial has provided preliminary results which demonstrate favourable changes in the faecal microbial profiles and severity of symptoms of IBS patients while being administered a synbiotic formulation containing VRI 003 and RS, or VRI 003 alone.

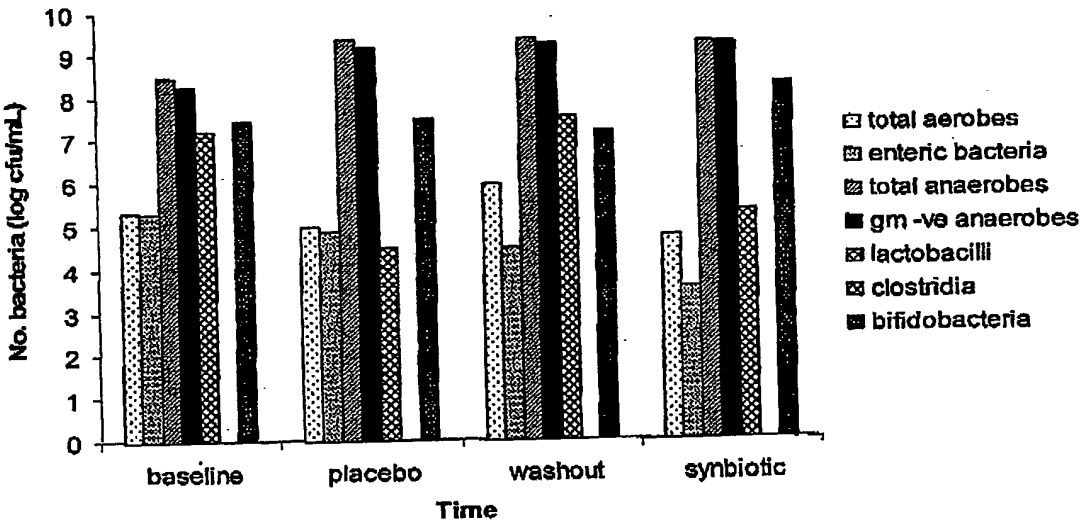
Although the invention has been described with reference to these specific examples, it will be appreciated by those skilled in the art that the invention may be embodied in many other forms.

Dated this 6th Day of September 2002

VRI BIOMEDICAL LIMITED

Attorney: IVAN A. RAJKOVIC
Fellow Institute of Patent and Trade Mark Attorneys of Australia
of BALDWIN SHELSTON WATERS

Fig. 1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baseline
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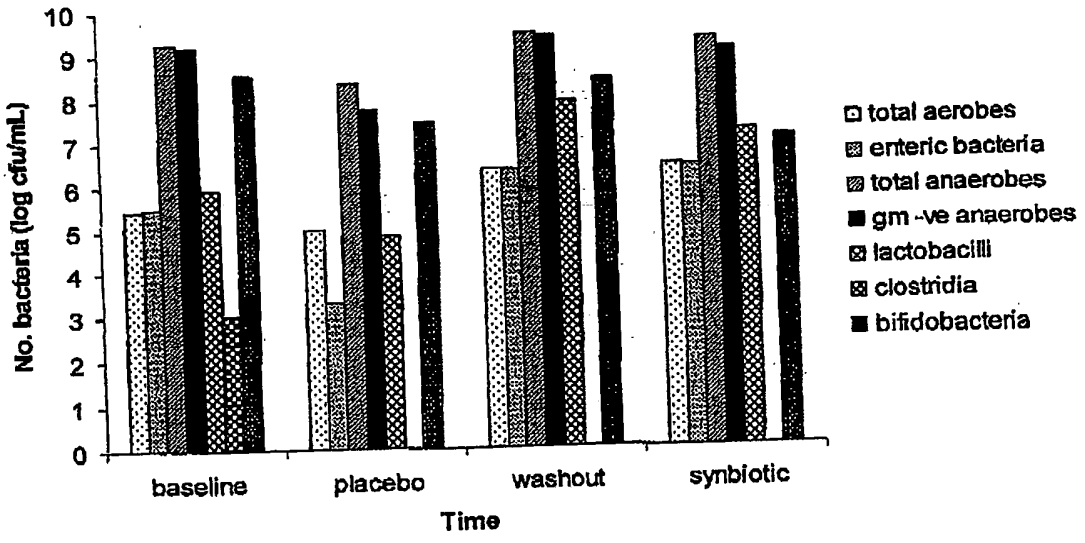
washout
synbiotic
Time
total aerobes
enteric bacteria
total anaerobes
gm -ve anaerobes
lactobacilli
clostridia
bifidobacteria

Fig. 2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baseline
placebo
washout
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Time
total aerobes
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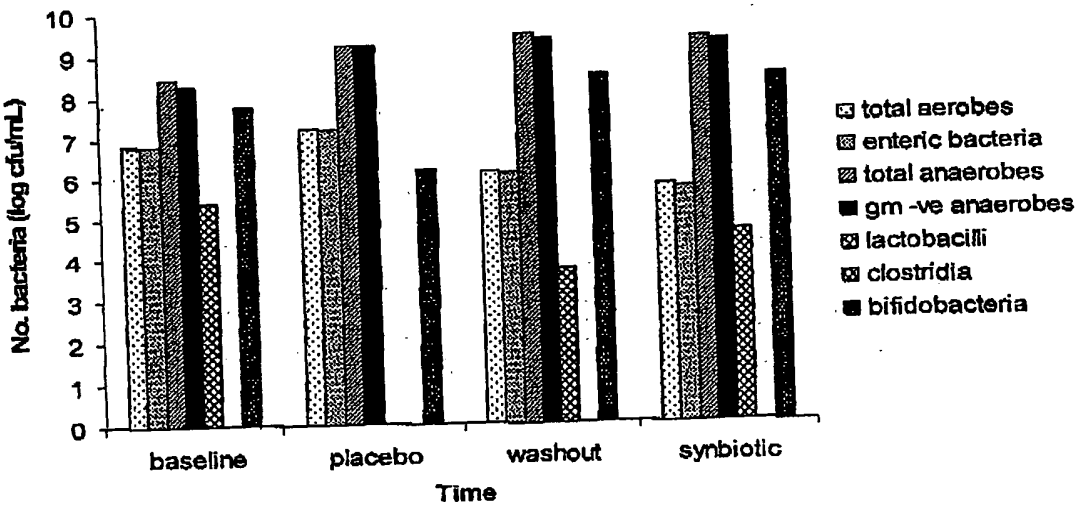
total anaerobes
gm -ve anaerobes
lactobacilli
clostridia
bifidobacteria

Fig. 3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baseline
placebo
washout
synbiotic
Time
total aerobes
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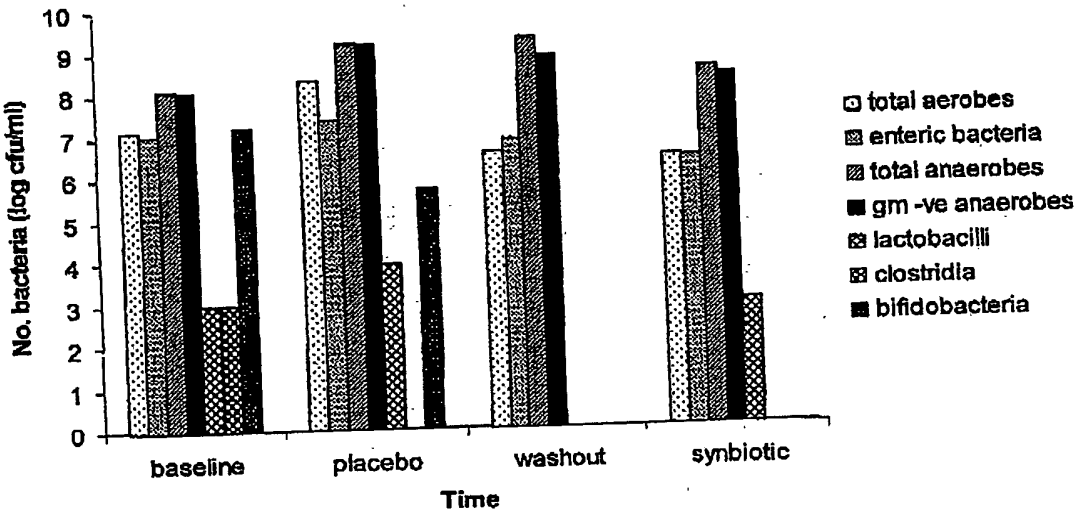
gm -ve anaerobes
lactobacilli
clostridia
bifidobacteria

Fig. 4


No. bacteria (log cfu/ml)
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8
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3
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1
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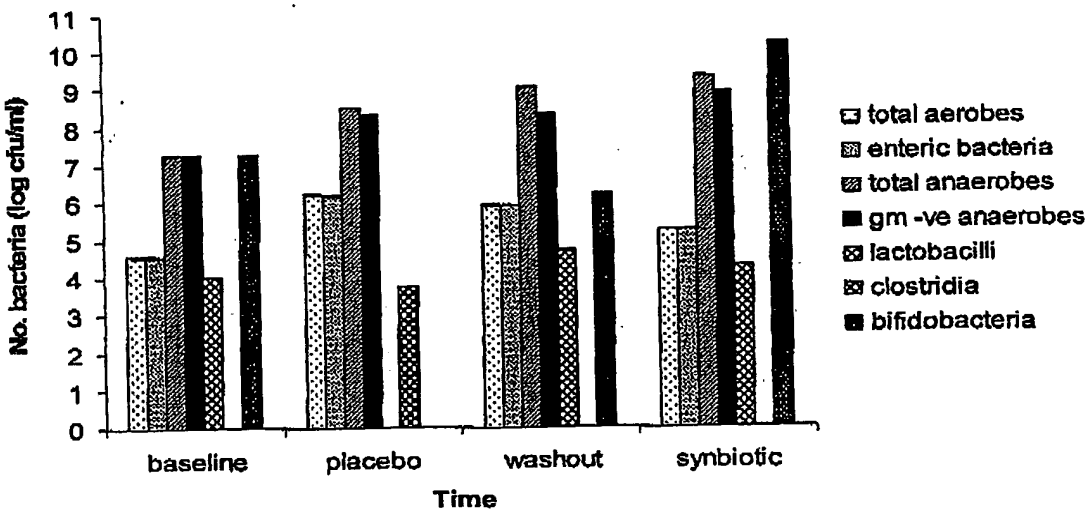
baseline
placebo
washout
synbiotic
Time
total aerobes
enteric bacteria
total anaerobes
gm -ve anaerobes
lactobacilli
clostridia
bifidobacteria

Fig. 5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7
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4
3
2
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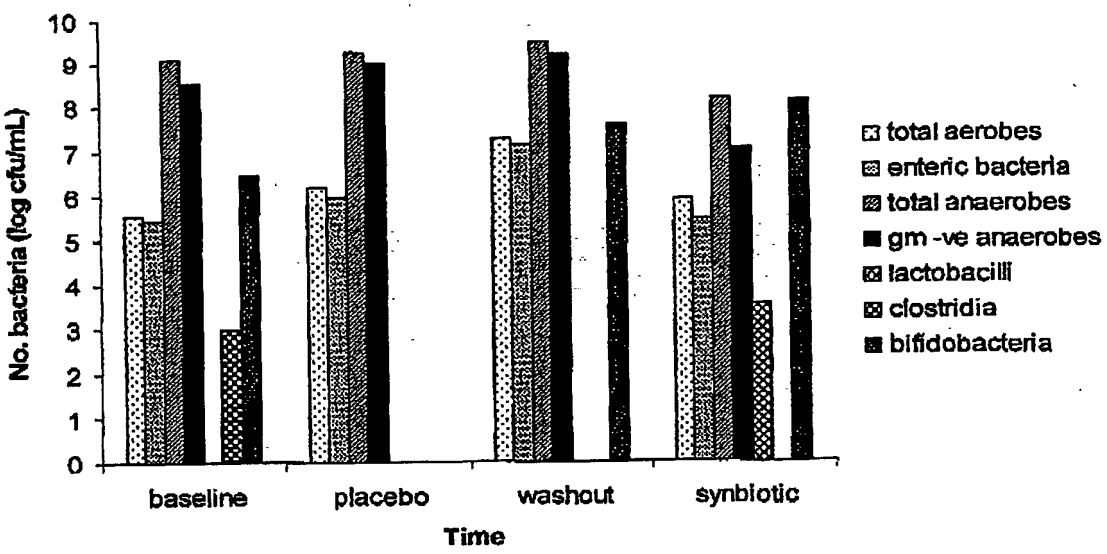
baseline
placebo
washout
synbiotic
Time
total aerobes
enteric bacteria
total anaerobes
gm -ve anaerobes
lactobacilli
clostridia
bifidobacteria

Fig. 6


No. bacteria (log cfu/mL)
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8
7
6
5
4
3
2
1
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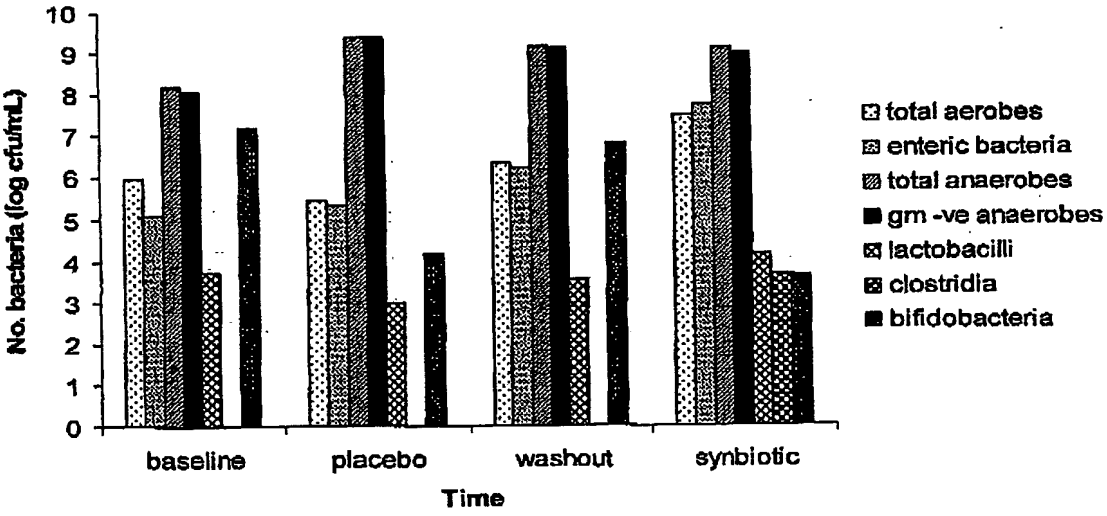
baseline
placebo
washout
synbiotic
Time
total aerobes
enteric bacteria
total anaerobes
gm -ve anaerobes
lactobacilli
clostridia
bifidobacteria

Fig. 7


No. bacteria (log cfu/mL)
10
9
8
7
6
5
4
3
2
1
0
baseline
placebo
washout
synbiotic
Time
total aerobes
enteric bacteria
total anaerobes
gm -ve anaerobes
lactobacilli
clostridia
bifidobacteria